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Short Communication

Effects of cAMP-Dependent Protein Kinase Activator and Inhibitor on In Vivo Rolipram Binding to Phosphodiesterase 4 in Conscious Rats

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ABSTRACT Rolipram is a selective inhibitor of phosphodiesterase-4 (PDE4), and positron emission tomography (PET) using [^{11}C]rolipram can monitor the in vivo activity of this enzyme that is part of the cAMP second messenger cascade. cAMP-dependent protein kinase (PKA) phosphorylates PDE4 and increases both enzyme activity and affinity for rolipram. In the present PET study, we examined effects of PKA modulators in conscious rats on the binding of [^{11}C](*R*)-rolipram in comparison to the much less active enantiomer [^{11}C](*S*)-rolipram. Unilateral injection of a PKA activator (dibutyryl-cAMP) and a PKA inhibitor (Rp-adenosine-3′,5′-cyclic monophosphorothioate) into the striatum significantly increased and decreased, respectively, the binding of [^{11}C](*R*)-rolipram. These effects were not caused by changes in blood flow or delivery of radioligand to brain, since these agents had no effect on the binding of [^{11}C](*S*)-rolipram binding. These results support the value of measuring in vivo [^{11}C](*R*)-rolipram binding in brain to assess responses to physiological or pharmacological challenges to the cAMP second messenger system. **Synapse 64:172–176, 2010.** Published 2009 Wiley-Liss, Inc.†

INTRODUCTION

Molecular imaging using radioligands and positron emission tomography (PET) is a valuable tool to study intact brain tissue both in living patients and in animal models of disease. However, relatively few radioligands have been developed, and almost all PET imaging has studied membrane-bound receptors and transporters but not intracellular signal transduction systems. The 3′,5′-cyclic adenosine monophosphate (cAMP) cascade is a major signal transduction system in brain and may be involved in psychiatric illnesses such as mood disorders (Duman et al., 1997) and drug addiction (Nestler and Aghajanian, 1997). cAMP is synthesized by adenylyl cyclase and is metabolized by phosphodiesterases (PDEs). PDE Type 4 (PDE4) is the major isozyme that hydrolyzes cAMP in brain. By ^{11}C-labeling rolipram, a selective inhibitor of PDE4, the cAMP cascade has been imaged with PET in rat (Fujita et al., 2005), monkey (Tsukada et al., 2001), and man (DaSilva et al., 2002; Matthews et al., 2003).

In addition to measuring an enzyme, PDE4, that plays a critical role in the cAMP cascade, [^{11}C](*R*)-rolipram may be able to monitor the in vivo phosphorylation status of this enzyme. cAMP-dependent protein kinase A (PKA) not only phosphorylates and activates PDE4 but also increases the sensitivity of PDE4 to inhibition by rolipram (Hoffmann et al.,

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1998). Thus, we predicted that activation of PKA would increase binding affinity and brain uptake of [^{11}C](*R*)-rolipram and that inhibition of PKA would have the opposite effects. We chose two drugs that are well known to modulate PKA's phosphorylation activity. PKA was activated with a cell permeable-cAMP analog, dibutyryl cyclic-AMP (db-cAMP), and PKA was inhibited with Rp-adenosine-3′,5′-cyclic monophosphorothioate (Rp-cAMPS). Unfortunately, these drugs have low permeability through the blood-brain barrier, and direct injection of drugs into striatum can have local effects on blood flow and uptake of the radioligand. To assess such local nonspecific effects, we also studied the much less active enantiomer [^{11}C](*S*)-rolipram. We previously showed that uptake of [^{11}C](*S*)-rolipram in rat brain is an accurate measure of the nonspecific uptake of [^{11}C](*R*)-rolipram (Fujita et al., 2005).

The strength of PET molecular imaging is the ability to study intact living tissue. In vivo phosphorylation status of proteins may not be retained after sampling tissue for in vitro assays. Our previous study (Itoh et al., 2009) indicated this possibility. In that study, we measured K_D of rolipram both in vivo and in vitro and found that in vitro K_D values were markedly greater than the in vivo values consistent with dephosphorylation of PDE4 during tissue homogenization. Therefore, by performing PET [^{11}C](*R*)-rolipram scans, we may be able to detect enzyme activity of PDE4 and hence the activity of the cAMP cascade in living subjects, which may be difficult to study after sampling tissue.

To study whether the in vivo binding of [^{11}C](*R*)-rolipram reflects the phosphorylation status and activity of PDE4, we measured the uptake of radioactivity in rat striatum after unilateral injection of an activator (db-cAMP) and an inhibitor (Rp-cAMPS) of PKA. We hypothesized that db-cAMP would increase and that Rp-cAMPS would decrease [^{11}C](*R*)-rolipram binding. We studied awake rats for two reasons. First, we previously found that anesthesia significantly increased the in vivo affinity of [^{11}C](*R*)-rolipram in rat brain, and we wished the current study to reflect the most common condition for human scans—i.e., awake. Second, anesthesia was previously found to block the effect of Rp-cAMPS in rat brain on glucose metabolism measured with PET (Hosoi et al., 2005).

MATERIALS AND METHODS

Preparation of conscious rats for PET scans

Male Sprague-Dawley rats (315 ± 30 g; Taconic Farms, Hudson, NY) were operated and trained as previously described (Itoh et al., 2009). In brief, using a stereotaxic apparatus (David Kopf Instrument, Tujunga, CA), an acrylic plate was permanently attached to the skull and stainless guide cannulas were implanted into the left and right striatum. The animals were acclimated to the whole body-holder for PET beginning the day after surgery. The rats were placed into the holder, and the head and jaw areas were fixed at a horizontal position. This training was performed 2 h per day for 10 days to acclimatize the rats to the apparatus and minimize the stress response during the PET scan.

Intrastriatal drug administration

Db-cAMP sodium salt (Sigma-Aldrich, St. Louis, MO) and Rp-cAMPS triethylammonium salt (Sigma-Aldrich) were dissolved in saline to the concentration of 10 mM of db-cAMP or 100 mM of Rp-cAMPS. A drug solution of 1 μl was infused into the left striatum through an infusion cannula (33-gauge, 3.5 mm longer than the guide cannula) three times at 2.5, 1.5 and 0.5 h before the PET scans. At the same time, 1 μl of saline solution was infused into the right striatum. Solutions were infused for 4 min at a flow rate of 0.25 μl min^{-1} using a microsyringe pump. The infusion cannula was kept in place for at least 3 min to prevent the reflux of drugs along the cannula track. After the infusions, rats were fixed to the whole body-holder and positioned in the PET gantry.

PET scans

[^{11}C](*R*) and [^{11}C](*S*)-rolipram were synthesized by ^{11}C-methylation of (*R*)- and (*S*)-*desmethyl*-rolipram, respectively, as previously described (Fujita et al., 2005). These radioligands were dissolved in saline (1.4 ± 0.1 ml). Thirty minutes after the last intrastriatal injection, rats were injected i.v. with either [^{11}C](*R*)-rolipram (13 PET scans; 6 for db-cAMP and 7 for Rp-cAMPS) or [^{11}C](*S*)-rolipram (13 PET scans; 6 for db-cAMP and 7 for Rp-cAMPS). The injected activity and specific activity of the radioligands were as follows: 61.4 ± 18.5 MBq and 108 ± 36 GBq/μmol for [^{11}C](*R*)-rolipram; 46.3 ± 9.0 MBq and 96 ± 40 GBq/μmol for [^{11}C](*S*)-rolipram. The PET data were acquired for 100 min by the Advanced Technology Laboratory Animal Scanner. PET data were reconstructed with 3D exact positioning ordered subset expectation maximization algorithm achieving a 1.7-mm full width half maximum at the center (Seidel et al., 2003). No scatter or attenuation correction was applied. The concentration of radioactivity in brain was expressed as standardized uptake value (SUV), which normalizes for injected activity and body weight, using the formula of SUV = (radioactivity in striatum per g/injected radioactivity) body weight (g). The cumulative radioactivity from 0 to 100 min was calculated by the trapezoid rule to obtain the area under the curve (AUC, in units of SUV min). Image analysis was performed with PMOD 2.95 (pixel-wise

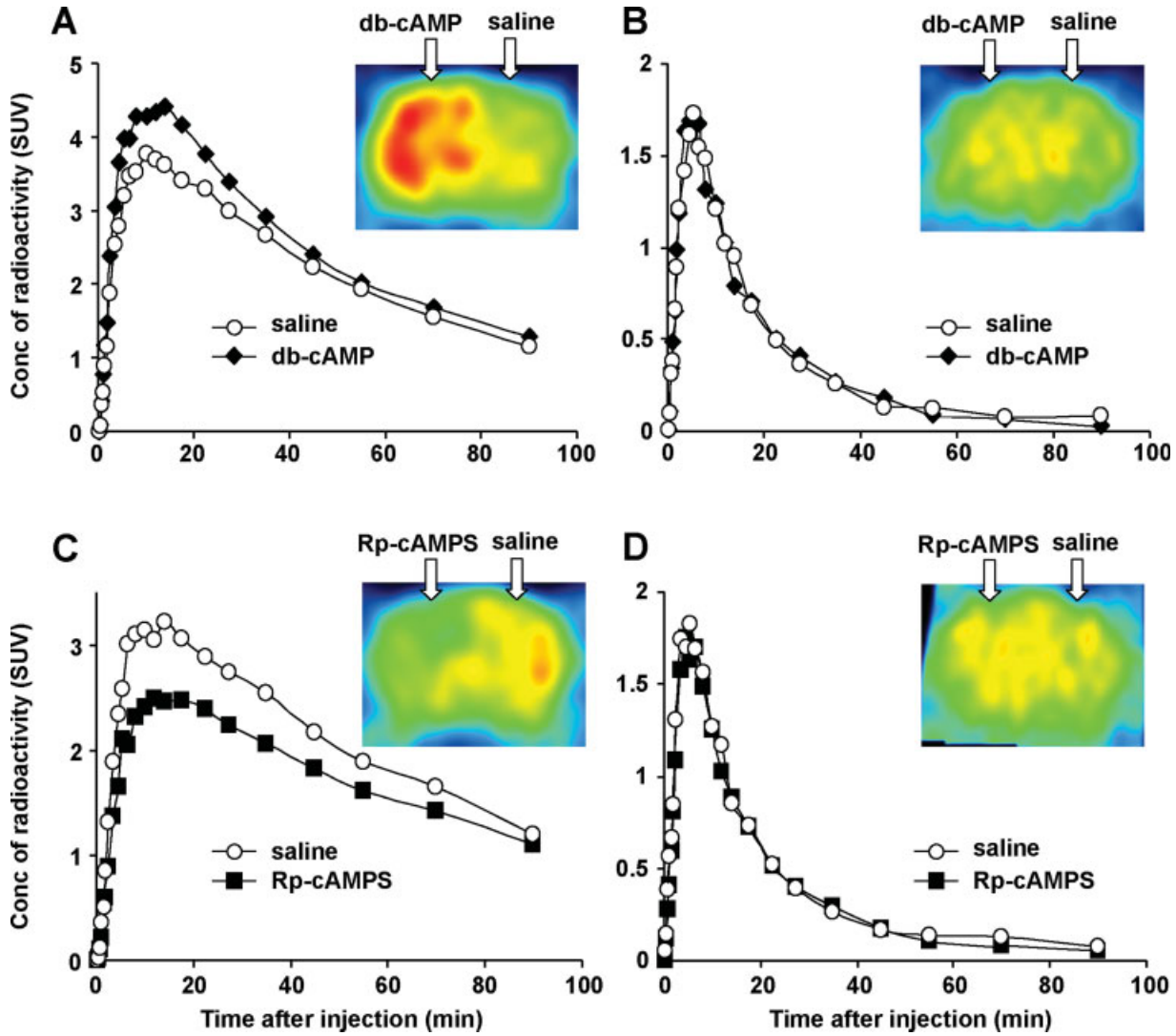

Fig. 1. Representative time-activity curves and PET images of the active enantiomer [^{11}C](*R*)-rolipram (**A**, **C**) and the less active enantiomer [^{11}C](*S*)-rolipram (**B**, **D**) binding measured with PET in rat striatum. Db-cAMP (A, B) or Rp-cAMPS (C, D) was administered into the left striatum three times before the PET scan. A value of 1 standardized uptake value (SUV) is equal to the concentration of radioligand that would be achieved if it were uniformly distributed in the body. Conc = concentration.

modeling software; PMOD Technologies, Zurich, Switzerland).

Statistical analysis

Data are expressed as mean ± SD. Parametric or nonparametric variables were determined by the Shapiro-Wilk normality test. Since all the variables were parametric, the statistical significance of differences was determined using paired *t* test. *P* values of less than 0.05 were considered to be statistically significant.

RESULTS AND DISCUSSION

In the current PET studies, [^{11}C](*R*)-rolipram had higher peak uptake and slower washout of radioactivity from brain than [^{11}C](*S*)-rolipram, and both characteristics are typical of specific binding by the more active *R*-enantiomer. [^{11}C](*R*)- and [^{11}C](*S*)-rolipram had peak concentrations of ~3 and ~1.5 SUV, respectively (Fig. 1). The concentration of radioactivity in brain decreased to half the peak value within ~50 and ~15 min for [^{11}C](*R*)- and [^{11}C](*S*)-rolipram, respectively. Based on our prior study with pharmacological displacements, [^{11}C](*R*)- and [^{11}C](*S*)-rolipram were used as measures of total and nondisplaceable (=nonspecific) binding, respectively (Fujita et al., 2005).

Injection of db-cAMP in all six animals increased the binding of [^{11}C](*R*)-rolipram in left striatum compared to the saline-injected right striatum (Fig. 1A). The $AUC_{0\text{-}100min}$ (SUV min) of the time-activity curve

TABLE I. Uptake of [11C]rolipram in rat striatum treated with PKA modulators

	$AUC_{0\text{-}100\ min}$ (SUV min)			
	[11C](*R*)-rolipram		[11C](*S*)-rolipram	
Drug	Drug	Saline	Drug	Saline
db-cAMP ($N = 6$)	175 ± 42*	158 ± 38	36 ± 14	36 ± 14
Rp-cAMPS ($N = 7$)	145 ± 23*	163 ± 30	36 ± 5	37 ± 6

Striatal uptake was measured as area under the curve (0–100 min) of the concentration of radioactivity in drug-treated (left) or saline-treated (right) striatum. Values are mean ± SD.
*$P < 0.01$ between drug and saline administration.

in left striatum was 10.8% larger ($P < 0.01$) than that in right striatum (Table I). In contrast, db-cAMP had no effect on striatal uptake after injection of [11C](*S*)-rolipram (Fig. 1B, Table I) indicating that the increase of [11C](*R*)-rolipram binding was caused by increased specific binding to PDE4 but not by other factors, such as increased permeability of the blood-brain barrier or increased cerebral blood flow.

The PKA inhibitor Rp-cAMPS decreased [11C](*R*)-rolipram binding in left compared to right striatum in all seven rats (Fig. 1C). The mean AUC of left striatum was 9.1% less than that in the right side ($P < 0.01$, Table I). In contrast, Rp-cAMPS had no effect on striatal uptake of radioactivity after injection of [11C](*S*)-rolipram (Fig. 1D, Table I).

In the current study, we sought to determine whether PET imaging with [11C](*R*)-rolipram would reflect the phosphorylation status of the enzyme PDE4, and we altered its phosphorylation status via PKA. In fact, a PKA activator (db-cAMP) increased and an inhibitor (Rp-cAMPS) decreased [11C](*R*)-rolipram binding in rat striatum compared to the contralateral side injected with saline. These two drugs did not alter the uptake of [11C](*S*)-rolipram, confirming that their effects were not mediated by nonspecific actions on local blood flow or permeability of the blood-brain barrier. These results strongly support, but do not prove, that uptake of [11C](*R*)-rolipram reflects the phosphorylation status of PDE4. Direct measurement of the phosphorylation state of the enzyme and the use of other drugs might add further evidence.

Our results are consistent with two of three prior studies of [11C](*R*)-rolipram in brain (Harada et al., 2002; Tsukada et al., 2001). Two studies in conscious monkeys measured both [11C](*R*)-rolipram and [11C](*S*)-rolipram binding in brain after intravenous injection of drugs active at the dopamine D_1 receptor, which increase intracellular concentrations of cAMP. Methamphetamine increases, and D_1 receptor antagonist SCH23390 decreases, [11C](*R*)-rolipram binding. These results are consistent with ours, since cAMP would stimulate PKA, which would then phosphorylate and activate PDE4. However, our results are inconsistent with an ex vivo study of [11C](*R*)-rolipram in rats after injection of dopaminergic agents (Lourenco et al., 2006). These inconsistent results from rats are highly suspected, because the authors did not correct for peripheral effects of radioligand delivery, they measured uptake at a single time point, and they did not use [11C](*S*)-rolipram to control for nonspecific effects.

An important methodological aspect of this PET study is our use of awake rats. We did so because we previously found that anesthesia affects the in vivo density and affinity of [11C](*R*)-rolipram binding (Itoh et al., 2009) and also found that anesthesia blocks the effect of Rp-cAMPS on cerebral metabolism measured with PET (Hosoi et al., 2005). Awake animals are clearly more difficult to scan than anesthetized ones. Nevertheless, to simulate typical conditions in humans, awake animals must be used when anesthesia has, or is likely to have, significant effects. Almost all prior PET studies on the effects of anesthesia have used monkeys, which are expensive and difficult to handle (Onoe et al., 1994). One important implication of the current work is that the less expensive and more easily handled rat can also be used for such investigations.

Uptake of [11C](*S*)-rolipram in rat brain reflects nonspecific binding, since it is equivalent to that of [11C](*R*)-rolipram with coadministration of saturating doses of nonradioactive (*R*)-rolipram (Fujita et al., 2005). Thus, [11C](*S*)-rolipram is useful to assess the nonspecific effects of drugs. For example, a relatively high dose of db-cAMP (25 nmol) increases cerebral blood flow (Hosoi et al., 2001). Although we used a lower dose (10 nmol) of db-cAMP, it still could have changed local cerebral blood flow and delivery of the radioligand. The less active enantiomer [11C](*S*)-rolipram was a valuable control to show that the effects of neither db-cAMP nor Rp-cAMPS were mediated by nonspecific effects of blood flow or radioligand delivery.

In conclusion, the intrastriatal injection in conscious rats of PKA activator and inhibitor significantly increased and decreased, respectively, the in vivo binding of [11C](*R*)-rolipram measured with PET. These alterations reflected specific binding of [11C](*R*)-rolipram to PDE4, since there were no effects on [11C](*S*)-rolipram uptake. These results provide strong evidence that [11C](*R*)-rolipram can monitor the in vivo activity and phosphorylation state of PDE4, an important regulatory enzyme in the cAMP second messenger cascade.

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